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**EVALUATION OF PHYSOSTIGMINE SALICYLATE
USING A HIGH CONCENTRATION OF LIVER S-9 FRACTION
IN THE AMES TEST FOR MUTAGENICITY**

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Don W. Korte, Jr., PhD, LTC, MSC*

**GENETIC TOXICOLOGY BRANCH
DIVISION OF TOXICOLOGY**

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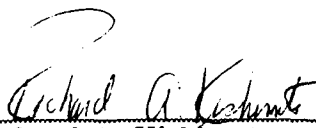
Evaluation of PHYSOSTIGMINE SALICYLATE Using a High Concentration of Liver S-9 Fraction in the Ames Test for Mutagenicity (Toxicology Series 229) --Sebastian and Korte

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Richard A. Kishimoto
COL, MSC
Acting Commander

13 July 1989
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ABSTRACT

The mutagenic potential of PHYSOSTIGMINE SALICYLATE was assessed in the Ames Test both in the presence and absence of a 10% liver S-9 activation mixture. Tester strains TA97, TA98, TA100, TA104, TA1535, TA1537, and TA1538 were exposed to doses ranging from 2×10^{-1} mg/plate to 6.4×10^{-5} mg/plate. The test compound was not mutagenic under the conditions of this test.

Key Words: Mutagenicity, Genetic Toxicology, Ames Test, Physostigmine salicylate, .

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PREFACE

TYPE REPORT: Ames Test GLP Study Report

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Project Officer: LTC Jurgen von Bredow, PhD, MSC

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GLP STUDY NUMBER: 88006

STUDY DIRECTOR: LTC Don W. Korte, Jr., PhD, MSC
Diplomate, American Board of Toxicology

PRINCIPAL INVESTIGATOR: Suzanne E. Sebastian, BA, SPC, USA

REPORT AND DATA MANAGEMENT:

A copy of the final report, study protocol, retired SOPs, stability and purity data on the test compound, and an aliquot of the test compound will be retained in the LAIR Archives.

TEST SUBSTANCE: PHYSOSTIGMINE SALICYLATE

INCLUSIVE STUDY DATES: 27 January - 4 February 1988

OBJECTIVE: The objective of this study was to determine the mutagenic potential of PHYSOSTIGMINE SALICYLATE by using the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test.

ACKNOWLEDGMENTS

SGT Lillie D. Witcher, BS, USA, SGT Gayle Orner, BS, USA, and SPC Joel Seewald, BS, USA, provided research assistance.

SIGNATURES OF PRINCIPAL SCIENTISTS INVOLVED IN THE
STUDY

We, the undersigned, declare that GLP Study 88006 was performed under our supervision, according to the procedures described herein, and that this report is an accurate record of the results obtained.

Don W. Korte, Jr. 12 July 89

DON W. KORTE, Jr, PHD / DATE
LTC, MSC
Study Director

Suzanne E. Sebastian 24 October 88

SUZANNE E. SEBASTIAN, BA / DATE
SPC, USA
Principal Investigator

Conrad R. Wheeler 27 Oct 88

CONRAD R. WHEELER, PhD / DATE
DAC
Analytical Chemist



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ATTENTION OF:

SGRD-ULZ-QA

12 July 1989

MEMORANDUM FOR RECORD

SUBJECT: GLP Compliance for GLP Study 88006

1. This is to certify that in relation to LAIR GLP Study 88006 the following inspections were made:

23 January 1988	- Protocol Review
02 February 1988	- Compound Preparation
02 February 1988	- Dosing

2. The institute report entitled "Evaluation of Physostigmine Salicylate using a High Concentration of Liver S-9 Fraction in the Ames Test of Mutagenicity," Toxicology Series 229 was audited on 13 June 1989.

Walter G. Bell

WALTER G. BELL
SFC, USA
Quality Assurance Auditor

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Evaluation of PHYSOSTIGMINE SALICYLATE Using a High Concentration of Liver S-9 Fraction in the Ames Test for Mutagenicity--Sebastian and Korte

INTRODUCTION

This laboratory has reported that physostigmine salicylate is not mutagenic in the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test (1). In this assay, a standard 4% concentration of liver S-9 fraction in the activated plates was used. It has been recommended that if a test compound is negative in the activated assay with the standard concentration of S-9, it should be retested at a 10% concentration of liver S-9 fraction (2). Since physostigmine produced a positive response in the mouse lymphoma assay (3), and sister chromatid exchange assays (4), a retest of physostigmine salicylate in the Ames Test was conducted using the recommended high concentration of liver S-9.

The Ames *Salmonella*/Mammalian Microsome Mutagenicity Test is a short-term screening test that utilizes histidine auxotrophic mutant strains of *Salmonella typhimurium* to detect compounds that are potentially mutagenic in mammals. A mammalian microsomal enzyme system is incorporated in the test to increase sensitivity by simulating *in vivo* metabolic activation of the test compound. The Ames test is an inexpensive yet highly predictive and reliable test for detecting mutagenic activity and thus carcinogenic potential (5).

This evaluation of PHYSOSTIGMINE SALICYLATE utilizes a revision of the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test (2).

Objective of the Study

The objective of this study was to determine the mutagenic potential of PHYSOSTIGMINE SALICYLATE in an Ames *Salmonella*/Mammalian Microsome Mutagenicity Test which utilized a concentration of 10% liver S-9 in the activation mixture.

MATERIALS AND METHODS

Test Compound

Chemical Name: PHYSOSTIGMINE SALICYLATE

LAIR Code Number: TW73

Physical State: White crystalline solid

Source: Division of Experimental Therapeutics
WRAIR, Washington, DC.

Requested by LTC Von Bredow, USAMRICD

Storage: PHYSOSTIGMINE SALICYLATE was stored in a desiccator at -20°C until used.

Chemical Properties/Analysis: Data provided by WRAIR characterizing the chemical composition and purity of the test material, are presented in Appendix A with a confirmatory analysis of the test material performed by the Division of Toxicology, LAIR (Presidio of San Francisco, CA).

Test Solvent

The positive control chemicals and the test compound were dissolved in grade I dimethyl sulfoxide (lot 113F-0450) obtained from Sigma Chemical Co. (St. Louis, MO). The glass-distilled water used in this assay was first passed through a Technic Series 300 Reverse Osmosis Unit (Seattle, WA), then through a Corning MP-1 Mega-Pure System glass distillation unit (Corning Glass Works, Corning, NY) (6).

Chemical Preparation

On the day of dosing, 300 mg of the test compound was measured into a sterile vial and dissolved in dimethyl sulfoxide to achieve a 10% (w/v) solution. Aliquots of this solution were used to dose the test plates.

Test Strains

Salmonella strains TA97, TA98, TA100, TA104, TA1535, TA1537, and TA1538 obtained directly from Dr. Bruce Ames, University of California, Berkeley, were used. These strains were maintained in our laboratory in liquid nitrogen. Quality control tests were run concurrently with the test substance to establish the validity of each strain's special features and to determine the spontaneous reversion rate. Descriptions of the strains, their genetic markers, and the

methods for strain validation are given in the LAIR SOP, OP-STX-1 (7).

Test Format

PHYSOSTIGMINE SALICYLATE was evaluated for mutagenic potential according to the revised Ames method (2). A detailed description of the methodology is given in LAIR SOP, OP-STX-1 (7).

Toxicity Tests

Toxicity tests were conducted to determine a sublethal concentration of the test substance (Table 1). This toxicity level was found by using minimal glucose agar (MGA) plates, concentrations of test compound ranging from 1.6×10^{-3} mg/plate to 5.0 mg/plate, and approximately 10^8 cells of TA100 per plate. Top agar containing trace amounts of histidine and biotin was placed on the plates. Strain verification was confirmed on the bacteria, along with a determination of the spontaneous reversion rate. After incubation, the growth on the plates was observed. Since the two highest doses showed a decrease in the number of macrocolonies (below the number in the spontaneous reversion plates) or an observable reduction in the density of the background lawn, a maximum limit dose of 0.2 mg/plate was used in the mutagenicity test.

Mutagenicity Test

The test substance was evaluated over a 1000-fold range of concentrations, decreasing from the minimum toxic level (the maximum or limit dose) by a dilution factor of 5, both with and without 0.5 ml of the S-9 microsome fraction. The S-9 was purchased from Microbiological Associates, Inc. (Bethesda, MD). After all the ingredients were added, the top agar was mixed, then overlaid on MGA plates. These plates contained 2% glucose and Vogel Bonner "E" Concentrate (8). The water used in this medium and in all reagents came from a Technic Model 301 Reverse Osmosis Pre-Treatment Water System (Seattle, WA) (6). Plates were incubated upside down in the dark at 37°C for 72 hours (Maron, 1985, personal communication). Plates were prepared in triplicate and the individual revertant counts were recorded. The average number of revertants at each dose level was compared to the average number of spontaneous revertants (negative control). The spontaneous reversion rate (with and without S-9) was monitored by averaging the counts from two determinations run simultaneously with the test compound. The spontaneous reversion rate was determined by inoculating one set of plates

before and one set after the test compound plates so that any change in spontaneous reversion rate during the dosing procedure would be detected. This spontaneous reversion rate was also compared with historical values for this laboratory and those cited in Maron and Ames (2). Sterility and strain verification controls were run concurrently. All reagents, test compounds, and media were checked for sterility by plating samples of each on minimal glucose agar and incubating them at 37°C with the test plates. The integrity of the different *Salmonella* strains used in the assay was verified by the following standard tests:

- Lack of growth (inhibition) in the presence of crystal violet which indicates that the prerequisite alteration of the lipopolysaccharide layer of the cell wall is present.
- Growth in the presence of ampicillin-impregnated disks which indicates the presence of an ampicillin-resistant R Factor in all strains except TA1535, TA1537 and TA1538.
- Lack of growth (inhibition) following exposure to ultraviolet light which indicates the absence of the DNA excision-repair mechanism

Three known mutagens were tested as positive controls to confirm the responsiveness of the strains to the mutation process. Each strain must be tested with at least one positive control but may be tested with several. These compounds: benzo[a]pyrene (lot 18C-0378), 2-aminofluorene (lot 021547), and N-methyl-N'-nitro-N-nitrosoguanidine (lot 127C-0342) were obtained from Sigma Chemical Co. (St. Louis, MO). The test compound and the known mutagens were handled during this study in accordance with the standards published in *NIH Guidelines for the Laboratory Use of Chemical Carcinogens* [DHHS Publication No. (NIH) 81-2385, May 1981].

Data Interpretation

According to Brusick (9), a compound is considered mutagenic if a positive dose response (correlated dose response) over three dose concentrations is achieved with at least the highest dose yielding a revertant colony count greater than or equal to twice the spontaneous colony count for the tester strains TA98, and TA100, or three times the spontaneous colony count for strains TA1535, TA1537, and TA1538 (5,7). A strong correlated dose response in strain TA100 without a doubling of the individual colony count may also be considered positive.

Maron and Ames (2) consider a compound mutagenic in tester strain TA97 if a correlated dose response over three concentrations is achieved with the highest dose yielding a revertant colony count greater than or equal to twice the spontaneous colony count.

Deviations from the Protocol/SOP

As indicated in the protocol, strain TA104 is used as a replacement for strain TA102. This permanent change will be reflected in the next revision of the Ames SOP.

Storage of the Raw Data and Final Report

A copy of the final report, study protocols, raw data, SOPs, and an aliquot of the test compound will be retained in the LAIR archives.

RESULTS

On 4 February 1988, the toxicity of PHYSOSTIGMINE SALICYLATE was determined (Table 1). For this experiment all sterility, strain verification, and negative controls were normal. Exposure of the tester strain (TA100) to the two highest doses showed a decrease in the number of macrocolonies, and an observable reduction in the density of the background lawn, indicating chemical toxicity. Therefore, the highest dose selected for the mutagenicity test was 0.2 mg/plate.

Normal results were obtained for all sterility and strain verification tests during the Ames Test performed on 4 February 1988, (Table 2). PHYSOSTIGMINE SALICYLATE did not induce any appreciable increase in the revertant colony counts relative to those of the negative control cultures (Table 3).

A tabular presentation of raw data is included in Appendix B.

TABLE 1: Toxicity Level Determination for TW73

GLP STUDY NUMBER 88006

TOXICITY DETERMINATION REVERTANT PLATE COUNT (TA100)

<u>CONCENTRATION</u>	<u>MEAN</u>	<u>± 1SD</u>	<u>BACKGROUND LAWN*</u>
START RUN NEGATIVE CONTROL	76	± 4.7	NL
5.0 mg/plate	0	± 0.0	ST
1.0 mg/plate	21	± 7.6	ST
0.2 mg/plate	84	± 10.6	NL
0.04 mg/plate	80	± 10.4	NL
0.008 mg/plate	86	± 6.2	NL
0.0016 mg/plate	71	± 7.8	NL
END RUN NEGATIVE CONTROL	91	± 7.2	NL

STRAIN VERIFICATION FOR TOXICITY DETERMINATION

TA100*

HISTIDINE REQUIREMENT	NG
AMPICILLIN RESISTANCE	G
UV	NG
CRYSTAL VIOLET SENSITIVITY	NG
STERILITY CONTROL	NG

STERILITY CONTROL FOR TOXICITY DETERMINATION

<u>MATERIAL TESTED</u>	<u>OBSERVATION*</u>
MINIMAL GLUCOSE AGAR PLATES	NG
TOP AGAR	NG
DILUENT WATER	NG
NUTRIENT BROTH	NG
TEST COMPOUND (HIGHEST DOSE)	NG

*NL=Normal Lawn, G=Growth, NG=No Growth, ST=Slight Toxicity.

**TABLE 2: Strain Verification and Sterility Testing
for the Mutagenicity Determination of TW73**

GLP STUDY NUMBER 88006

<u>STRAIN VERIFICATION</u>					
<u>OBSERVATIONS*</u>					
<u>STRAIN</u>	<u>HISTIDINE REQUIREMENT</u>	<u>AMPICILLIN RESISTANCE</u>	<u>UV REPAIR</u>	<u>CRYSTAL VIOLET</u>	<u>STERILITY CONTROL</u>
TA97	NG	R	NG	NG	NG
TA98	NG	R	NG	NG	NG
TA100	NG	R	NG	NG	NG
TA104	NG	R	NG	NG	NG
TA1535	NG	NR	NG	NG	NG
TA1537	NG	NR	NG	NG	NG
TA1538	NG	NR	NG	NG	NG

<u>STERILITY CONTROL FOR MUTAGENICITY DETERMINATION</u>	
<u>MATERIAL TESTED</u>	<u>OBSERVATION*</u>
MINIMAL GLUCOSE AGAR PLATES	NG
TOP AGAR	NG
DILUENT WATER	NG
NUTRIENT BROTH	NG
TEST COMPOUND (HIGHEST DOSE)	NG
S-9	NG

* G=Growth, NG=No Growth, R=Resistant, NR=Not Resistant

TABLE 3: Mutagenicity Assay for Physostigmine Salicylate (TW73)†

COMPOUND*	DOSE/PLATE	TA97	TA98	TA100	TA104
WITHOUT S-2					
NEG CONTROL	0.0 mg	66 ± 23.3	17 ± 2.7	66 ± 13.8	138 ± 28.0
MNNG	2.0 µg	209 ± 10.1	-	866 ± 37.2	922 ± 46.9
TW73	0.2 mg	101 ± 5.1	18 ± 1.7	73 ± 17.0	189 ± 10.1
TW73	0.04 mg	69 ± 14.4	14 ± 4.2	79 ± 10.6	141 ± 20.1
TW73	0.008 mg	72 ± 10.0	15 ± 3.0	74 ± 12.6	151 ± 13.0
TW73	0.0016 mg	79 ± 8.1	11 ± 3.5	63 ± 6.6	169 ± 11.3
TW73	0.00032 mg	92 ± 7.4	15 ± 1.5	79 ± 10.8	193 ± 16.8
TW73	0.000064 mg	82 ± 8.2	17 ± 9.9	77 ± 18.9	187 ± 10.5
WITH S-2					
NEG CONTROL	0.0 mg	107 ± 14.7	28 ± 4.7	85 ± 8.6	207 ± 26.4
2-AF	2.0 µg	210 ± 2.5	-	-	-
BP	2.0 µg	-	80 ± 2.3	226 ± 18.3	-
TW73	0.2 mg	88 ± 6.4	26 ± 4.6	88 ± 4.5	204 ± 12.5
TW73	0.04 mg	93 ± 11.0	26 ± 2.3	89 ± 10.8	180 ± 3.6
TW73	0.008 mg	98 ± 6.8	23 ± 5.6	73 ± 9.0	235 ± 10.4
TW73	0.0016 mg	87 ± 9.8	22 ± 4.4	88 ± 5.0	214 ± 5.8
TW73	0.00032 mg	98 ± 7.8	23 ± 5.2	75 ± 6.7	227 ± 12.1
TW73	0.000064 mg	93 ± 7.0	22 ± 3.5	83 ± 6.0	249 ± 1.5

† Values represent the mean number of revertants/plate (±standard deviation).

* 2-AF=2-aminofluorene, BP=benzo[a]pyrene, MNNG=N-methyl-N'-nitro-N-nitrosoguanidine.

TABLE 3 (cont.): Mutagenicity Assay for Physostigmine Salicylate (TW73)†

COMPOUND*	DOSE/PLATE	TA1535	TA1537	TA1538
WITHOUT S-2				
NEG CONTROL	0.0 mg	20 ± 3.7	5 ± 3.8	6 ± 4.4
MNNG	2.0 µg	773 ± 31.6	-	-
TW73	0.2 mg	13 ± 3.1	9 ± 2.3	25 ± 1.2
TW73	0.04 mg	10 ± 2.3	2 ± 1.5	14 ± 10.2
TW73	0.008 mg	10 ± 2.1	5 ± 1.7	6 ± 6.5
TW73	0.0016 mg	16 ± 1.5	2 ± 0.6	6 ± 4.7
TW73	0.00032 mg	18 ± 6.2	6 ± 2.0	16 ± 16.5
TW73	0.000064 mg	11 ± 3.1	4 ± 2.6	6 ± 2.1
WITH S-2				
NEG CONTROL	0.0 mg	18 ± 3.5	12 ± 3.0	19 ± 3.1
2-AF	2.0 µg	-	-	-
BP	2.0 µg	-	31 ± 9.2	145 ± 16.2
TW73	0.2 mg	19 ± 5.9	9 ± 2.3	4 ± 1.5
TW73	0.04 mg	9 ± 5.3	5 ± 0.7	7 ± 4.7
TW73	0.008 mg	6 ± 3.2	3 ± 1.5	13 ± 3.6
TW73	0.0016 mg	15 ± 4.5	4 ± 1.7	13 ± 5.3
TW73	0.00032 mg	11 ± 2.0	6 ± 1.0	17 ± 1.5
TW73	0.000064 mg	16 ± 3.6	9 ± 5.2	15 ± 4.2

† Values represent the mean number of revertants/plate (± standard deviation).

* 2-AF=2-aminofluorene, BP=benzo[a]pyrene, MNNG=N-methyl-N'-nitro-N-nitrosoguanidine.

DISCUSSION

Certain test criteria must be satisfied before an Ames test can be considered a valid assessment of a compound's mutagenic potential. First, the special features of the Ames strains must be verified. These features include demonstration of ampicillin resistance, alterations in the LP layer, and deficiency in DNA excision-repair. Second, the *Salmonella* strains must be susceptible to mutation by known mutagens. Third, the optimal concentration of the test compound must be determined by treating TA100 with a broad range of doses and observing the potential toxic effects on formation of macrocolonies and microcolonies. If these tests are performed and expected data are obtained, then the results of an Ames test can be considered valid.

Since physostigmine salicylate was more potent after metabolic activation in mammalian cell assays for genotoxic potential (3,4) but was not active in the Ames test that used a standard 4% concentration of liver S-9 fraction in the activation mixture (1), a repeat of the Ames test was conducted with a higher concentration of liver S-9. Even at the recommended concentration of 10% liver S-9 fraction in the activation mixture (2), physostigmine salicylate was not active in the Ames Test. These data suggest that the genotoxic potential of physostigmine salicylate is due to chromosomal mutations as detected in the mouse lymphoma or sister chromatid exchange assays rather than gene mutations as would be detected in the Ames test.

CONCLUSION

PHYSOSTIGMINE SALICYLATE was evaluated for mutagenic potential in the Ames Test, both in the presence and absence of a 10% liver S-9 activation mixture, and did not induce a positive response under conditions of this study.

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APPENDICES

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Appendix A: CHEMICAL DATA

Chemical name: Physostigmine salicylate

Other Names: Eserine salicylate; physostigmine, 2-hydroxybenzoate; 1, 2, 3, 3a, 8, 8a-hexahydro-1, 3a, 8-trimethylpyrrolo[2,3-b]indol-5-ol methylcarbamate (ester), (3aS-cis)-, mono (2-hydroxybenzoate) (salt)

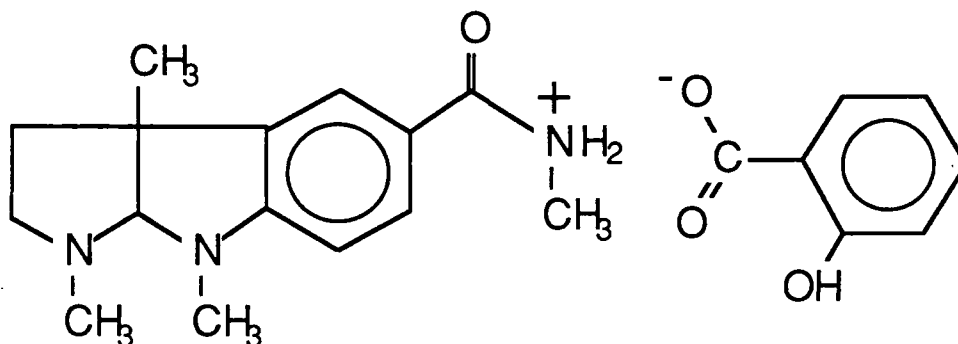
Lot Number: BL25591

Chemical Abstracts Registry Number: 57-64-7

LAIR Code Number: TW73

WRAIR Code Number: WR-006570AM

Chemical Structure:



Molecular Formula: $C_{15}H_{21}N_3O_2 \cdot C_7H_6O_3$

Molecular Weight: 413.47

Physical State: White, crystalline solid

Analytical Data:

The test compound was analyzed by the sponsors and the identity confirmed by UV and IR spectroscopy, high pressure liquid chromatography, mass spectrometry and elemental analysis.¹ Based on HPLC analysis of this test compound in comparison with the USP physostigmine salicylate reference standard, lot BL25591 contains 66.7% (100.1% of theory) physostigmine base and 33.7% (100.8% of theory) salicylic acid or 100.4% physostigmine salicylate.¹

HPLC: HPLC analysis of physostigmine salicylate in this lab was performed using a Hewlett-Packard 1090 HPLC system equipped with a diode array detector. The compound was chromatographed under the following conditions: silica column (4.6 x 100 mm, Brownlee Labs, Inc.); mobile phase, 15%

Appendix A (cont.): CHEMICAL DATA

acetonitrile/buffer (0.01M Na₂HPO₄ with 0.0025 M tetramethylammonium chloride); flow rate, 1.5 ml/min; column oven, 50°C; wavelength monitored, 210 nm. The compound eluted as two peaks with retention times of 0.9 min (salicylic acid), and 3.9 min (physostigmine).²

IR (KBr): 3320(broad), 2964, 2325, 1744, 1629, 1594, 1485, 1460, 1383, 1326, 1245, 1203, 1184, 1151, 1140, 1087, 1006, 993, 944, 860, 807, 754, 704, 667, 382 cm⁻¹.³ The IR spectrum was identical to that provided by the sponsors.¹

Source: Mr. William Ellis

Division of Experimental Therapeutics
Walter Reed Army Institute of Research
Washington, DC

Requested by LTC Jurgen von Bredow, PhD, MSC

¹ Masamori E, Benitez A, and Lim P. Assay of physostigmine salicylate, WR-6570AM, BL25591. Menlo Park, CA: SRI International, 4 November 1986; Report no. 553.

² Wheeler CR. Toxicity testing of antidotes of chemical warfare agents. Laboratory notebook #85-12-024.6, pp 2-11. Letterman Army Institute of Research, Presidio of San Francisco, CA.

³ Wheeler CR. Toxicity testing of antidotes of chemical warfare agents. Laboratory notebook #85-12-024.3, pp 10-13. Letterman Army Institute of Research, Presidio of San Francisco, CA.

Appendix B: INDIVIDUAL PLATE SCORES

PHYSOSTIGMINE SALICYLATE (TW73) TOXICITY DETERMINATION WITH TA100

DOSES	5.0 mg/plate	1.0 mg/plate	0.2 mg/plate	0.04 mg/plate
PLATE 1	0	30	86	88
PLATE 2	0	16	73	83
PLATE 3	0	18	94	68
BACKGROUND	sl. toxicity	sl. toxicity	normal lawn	normal lawn

DOSES	0.008 mg/plate	0.0016 mg/plate	NEG START	NEG END
PLATE 1	88	76	81	99
PLATE 2	91	65	74	86
PLATE 3	79	-	72	87
BACKGROUND	normal lawn	normal lawn	normal lawn	normal lawn

Appendix B (cont.): INDIVIDUAL PLATE SCORES

 PHYSOSTIGMINE SALICYLATE (TW73)
 NEGATIVE CONTROL DATA

COMPOUND	DOSE/PLATE	TA97	TA98	TA100	TA104	TA1535	TA1537	TA1538
<u>WITHOUT S-9</u>								
NEG CONTROL	0.0 mg	71	18	86	163	20	10	10
(START RUN)		100	18	51	159	14	7	8
		85	14	77	166	20	8	10
NEG CONTROL	0.0 mg	44	18	52	121	17	4	1
(END RUN)		46	13	64	120	23	2	0
		49	20	66	100	24	0	5
<u>WITH S-9</u>								
NEG CONTROL	0.0 mg	105	33	82	245	15	13	19
(START RUN)		115	25	93	225	14	8	24
		130	35	94	198	16	14	17
NEG CONTROL	0.0 mg	86	25	71	175	20	15	19
(END RUN)		106	24	83	214	20	12	21
		101	27	89	183	23	8	15

Appendix B (cont.): INDIVIDUAL PLATE SCORES

PHYSOSTIGMINE SALICYLATE (TW73)

POSITIVE CONTROL DATA

COMPOUND†	DOSE/PLATE	TA97	TA98	TA100	TA104	TA1535	TA1537	TA1538
2-AF	2.0 µg	210						
		207						
		212						
BP	2.0 µg		79	247	523		36	148
			83	212	531		20	128
			79	220	517		36	160
MNNG	2.0 µg	207		905	961	799		
		220		861	935	738		
		200		831	870	783		

† 2-AF=2-aminofluorene, BP=benzo[a]pyrene, MNNG=N-methyl-N'-nitro-N-nitrosoguanidine.

Appendix B (cont.): INDIVIDUAL PLATE SCORES

PHYSOSTIGMINE SALICYLATE (TW73)
 MUTAGENICITY DATA WITHOUT S-9

COMPOUND	DOSE/PLATE	TA97	TA98	TA100	TA104	TA1535	TA1537	TA1538
TW73	0.2 mg	97	16	53	183	10	10	24
		107	19	82	201	14	6	26
		100	19	83	184	16	10	24
TW73	0.04 mg	57	11	90	147	9	2	26
		65	13	69	158	13	4	7
		85	19	77	119	9	1	10
TW73	0.008 mg	64	15	72	150	8	6	13
		83	18	62	164	12	3	0
		68	12	87	138	9	6	6
TW73	0.0016 mg	74	15	64	175	14	1	11
		74	8	69	156	17	2	4
		88	11	56	176	16	2	2
TW73	0.00032 mg	84	16	88	184	23	8	35
		95	15	67	182	11	4	5
		98	13	82	212	20	6	8
TW73	0.000064 mg	73	10	84	198	12	3	5
		84	12	92	187	14	2	8
		89	28	56	177	8	7	4

Appendix B (cont.): INDIVIDUAL PLATE SCORES

PHYSOSTIGMINE SALICYLATE (TW73)
MUTAGENICITY DATA WITH S-9

COMPOUND	DOSE/PLATE	TA97	TA98	TA100	TA104	TA1535	TA1537	TA1538
TW73	0.2 mg	85	31	88	213	15	12	4
		95	22	93	190	26	8	3
		83	25	84	210	17	8	6
TW73	0.04 mg	87	23	81	179	7		12
		87	27	101	177	5	5	3
		106	27	84	184	15	4	5
TW73	0.008 mg	93	22	82	247	5	2	17
		106	29	64	229	10	3	12
		96	18	74	229	4	5	10
TW73	0.0016 mg	98	24	83	217	19	3	9
		81	25	93	207	10	3	19
		81	17	88	217	15	6	11
TW73	0.00032 mg	89	26	73	213	9	5	17
		102	17	82	234	11	7	15
		103	26	69	234	13	6	18
TW73	0.000064 mg	93	26	82	250	20	15	12
		86	20	89	247	13	6	14
		100	20	77	249	15	6	20

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